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## Rescue strategies in Drosophila models of neurodegenerative diseases

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# Chapter 2

Overexpression of cystathionine  $\gamma$ -lyase  
suppresses detrimental effects of  
spinocerebellar ataxia type 3

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## SUMMARY

Spinocerebellar ataxia type 3 (SCA3) is a polyglutamine (polyQ) disorder caused by a CAG repeat expansion in the *ATXN3* gene resulting in toxic protein aggregation. Inflammation and oxidative stress are considered secondary factors contributing to the progression of this neurodegenerative disease. There is no cure that halts or reverses the progressive neurodegeneration of SCA3. Here we show that overexpression of cystathionine  $\gamma$ -lyase, a central enzyme in cysteine metabolism, is protective in a *Drosophila* model for SCA3. SCA3 flies show eye degeneration, increased oxidative stress, insoluble protein aggregates, reduced levels of protein persulfidation and increased activation of the innate immune response. Overexpression of *Drosophila* cystathionine  $\gamma$ -lyase restores protein persulfidation, decreases oxidative stress, dampens the immune response and improves SCA3-associated tissue degeneration. Levels of insoluble protein aggregates are not altered; therefore the data implicate a modifying role of cystathionine  $\gamma$ -lyase in ameliorating the downstream consequences of protein aggregation leading to protection against SCA3-induced tissue degeneration. The cystathionine  $\gamma$ -lyase expression is decreased in affected brain tissue of SCA3 patients, suggesting that enhancers of cystathionine  $\gamma$ -lyase expression or activity are attractive candidates for future therapies.

## INTRODUCTION

Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease, is a rare progressive neurodegenerative disease and the most common dominantly inherited ataxia worldwide. SCA3 is a polyglutamine (polyQ) disorder caused by a CAG-trinucleotide repeat expansion encoding glutamine within the sequence of the *ATXN3* gene. The length of the repeat expansion is directly related to the aggregation propensity of the ataxin3 protein and is inversely related to the age of onset of the disease. Protein aggregates are considered to be the cause for neuronal dysfunction and death, which is supported by several lines of evidence showing that aggregate prevention or increased (autophagic) clearance, delays neuronal death and degeneration in multiple model systems<sup>1-4</sup>.

The pathophysiological sequel of neurodegeneration in SCA3 is not fully understood, although proteotoxic stress, transcriptional dysregulation, mitochondrial dysfunction, oxidative stress and inflammation have been implicated<sup>5-7</sup>. To date, there are no disease-modifying treatments for polyQ diseases like SCA3.

Cystathionine  $\gamma$ -lyase (CSE) is one of the central enzymes in cysteine and hydrogen sulfide metabolism ( $H_2S$ ). Homocysteine is a substrate for CSE leading to the production of  $H_2S$ ,  $\alpha$ -ketobutyrate, ammonia, homolanthionine and cystathionine, the latter serves as a CSE substrate to produce cysteine<sup>8,9</sup>. Cysteine is also a substrate for CSE leading to the production of  $H_2S$ , cystathionine and pyruvate<sup>8,9</sup>.  $H_2S$  and CSE are linked to aging and age-related pathologies<sup>10-13</sup>.  $H_2S$  can act as an endogenous modulator of oxidative stress either by direct scavenging of reactive oxygen species (ROS) and nitrogen species<sup>14</sup> or through increasing the intracellular glutathione (GSH) pool<sup>15,16</sup>.  $H_2S$  also confers cytoprotection via suppression of inflammation<sup>17</sup> and by protecting mitochondrial function and integrity<sup>17,18</sup>. Decreased levels of  $H_2S$  in brain tissue are associated with neurodegenerative age-related diseases like Parkinson's<sup>19</sup> and administration of  $H_2S$  has been shown protective in experimental models for this disease<sup>20-22</sup>. Decreased levels of CSE have recently been observed in human Huntington's disease tissues and in a mouse Huntington model<sup>22</sup>. After addition of sodium hydrogen sulfide and L-cysteine, levels of protein persulfidation (also referred to as protein S-sulfhydration) increased in a CSE dependent manner *in vitro*<sup>23</sup>, suggesting an influential effect of this type of posttranslational protein modification. Indeed, protein persulfidation has been demonstrated to mediate the activity of parkin, to serve as an antioxidant, and to protect against cellular senescence<sup>24-26</sup>. A possible link between SCA3, CSE and protein persulfidation remains to be determined as well as the potential neuroprotective effects of overexpressing the CSE enzyme directly in a neurodegenerative background.

Here, we investigated the possible protective role of CSE in a *Drosophila* model for SCA3. *Drosophila* was chosen because CSE is highly conserved between humans and flies (<http://flybase.org/blast>) and an established *Drosophila* model for SCA3 is available<sup>27,28</sup>. In the fly model for SCA3 a truncated version of the pathogenic human *ATXN3* gene containing a multiple CAG repeat is expressed and key features of SCA3 disease are present<sup>27-29</sup>. This is a suitable model because the CAG repeat, and not the mutated protein, is considered to be the disease causing entity in SCA3 and in several other polyQ diseases as well<sup>29,30</sup>. We used the *Drosophila* SCA3 model (also referred to as SCA3 flies) to investigate the effects of

CSE overexpression. We identified additional phenotypes in the SCA3 model such as, loss of tissue integrity in degenerative patches in the fly eye, increased activation of the innate immune response and decreased protein persulfidation. We found that transgene-mediated increased expression of CSE rescues these novel and also previously reported phenotypes of the fly SCA3 model. Rescue was also observed after addition of sodium thiosulfate, an FDA approved drug and a component of the transsulfuration pathway, in which CSE plays a central role<sup>9,31</sup>. The CSE mediated rescue occurs independently of protein aggregate formation, indicating that rescue effects occur downstream of the formation of these toxic entities. We also found endogenous expression of CSE in brain areas that are affected in SCA3 patients. In addition, a lower expression was observed in patient samples compared to controls. We present and discuss a possible role for CSE in polyQ disease pathology and treatment.

## MATERIALS AND METHODS

Below we provide a brief overview of the methods used for experiments presented in this article. For further details, please see *Supplementary Materials and Methods*.

### *Drosophila* stocks

As wild-type control, the  $\gamma$ 1w118 *Drosophila* line was used. Eip55E (*Drosophila* CSE)-overexpressing lines were generated in the laboratory. The *GMR-GAL4 UAS-SCA3trQ78* fly stock was a kind gift from Prof. Bonini<sup>28,32</sup>. The detailed description of the *Drosophila* lines and fly food, backcrossing and supplementation of chemical compounds information can be found in *Supplementary Materials and Methods*.

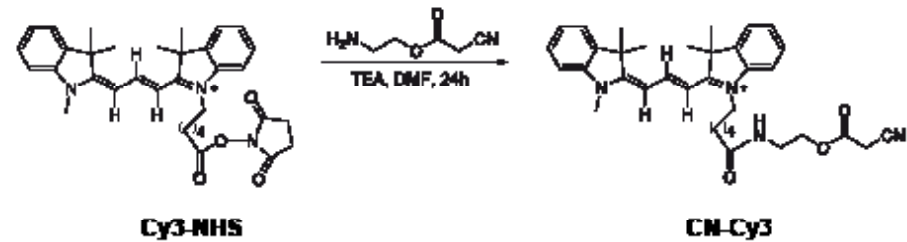
### Eye degeneration assay

To evaluate relative degeneration percentage, we used an eye scoring method that was previously described<sup>68, 69</sup>. Irregularly structured depigmented eyes without dark patches were defined as rough. The presence of one or more black patches along with the irregular structure and depigmentation was considered a degenerated rough eye. Each eye of one-day-old flies was scored as a singular entity. We scored the total amount of degenerated eyes as opposed to the total amount of eyes (rough + degenerated). Total count of eyes scored per condition was between 100 and 1000 depending on the number of progeny of a particular genotype.

### Protein persulfidation assay

To detect protein persulfidation, a tag-switch assay has been used as previously described<sup>70, 71</sup>. Briefly, protein extracts were prepared from either whole flies (3 flies per sample, 100 $\mu$ l of extraction buffer) for persulfidation analysis of CSE overexpression effect under control of actin driver in non-disease background or fly heads (25 heads per sample, 65 $\mu$ l of extraction buffer) for persulfidation analysis of CSE overexpression effect under control of GMR driver in SCA3 background. Extracts were prepared using

HEN buffer (50mM HEPES, 2mM EDTA, 1% NP-40) additionally supplemented with 2% SDS, 1% protease inhibitors and 20mM MSBT-A, a water-soluble methylsulfonyl benzothiazole derivate<sup>72</sup>. The extracts were incubated for 30 min on ice and 30 min at 37 °C. Proteins were then purified using water/methanol/chloroform precipitation (4/4/1, v/v/v). Samples were redissolved in PBS containing 2% SDS and treated with CN-biotin (Figure 4A) overnight. Protein concentrations were adjusted to the same value before the SDS electrophoresis was performed. Biotinylation of the samples was detected by Western blot, using anti-biotin antibodies (Sigma Aldrich). To improve the assay, we used a new probe, **CN-Cy3**, which served as an alternative for CN-biotin and allowed direct in-gel fluorescence measurements. **CN-Cy3** is a cyanoacetate derivative of a cyanine-based Cy3 fluorescence dye.



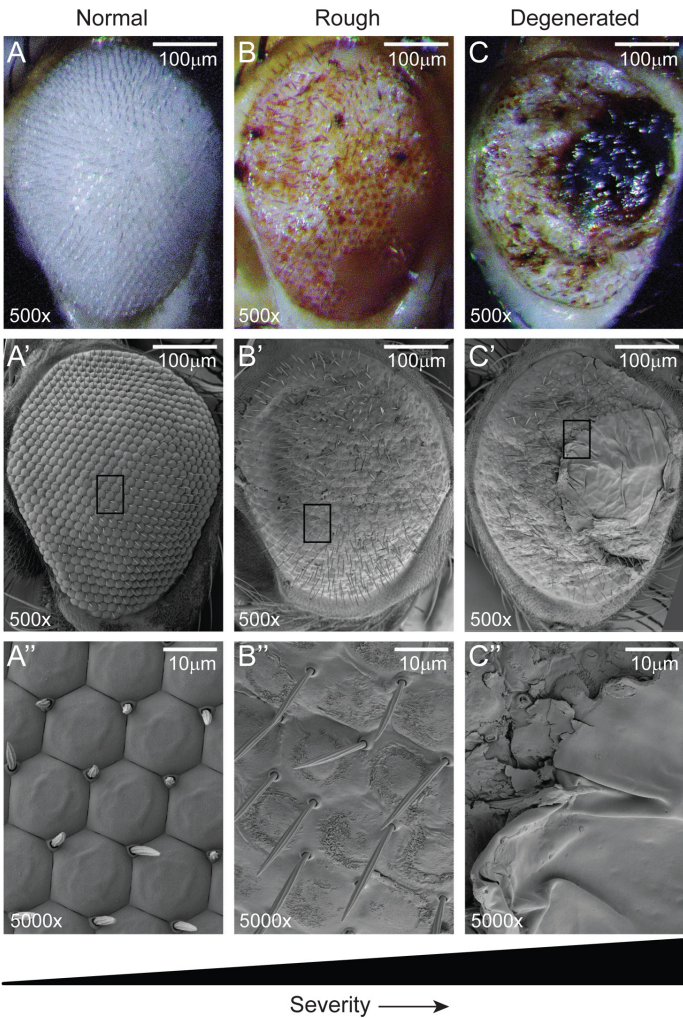
Molecular biology techniques

For the detailed description of quantitative RT-PCR, Western blot and Protein oxidation analyses, Immunohistochemistry that are used in the current study, please see *Supplementary Materials and Methods*.

RESULTS

SCA3 flies show increased tissue degeneration

A *Drosophila* model was used to further investigate a possible protective role of overexpression of CSE in SCA3. First, we performed an extended phenotypic analysis of this model. Previously, it has been shown that flies bearing *UAS-SCA3trQ78* – an inducible truncated version of the human *ATXN3* gene containing 78 CAG repeats – under the control of the glass multiple reporter (GMR) driver (also referred to as *GMR-GAL4-UAS-SCA3trQ78* or *SCA3* flies) develop progressive cellular eye degeneration. These flies develop a fully penetrant ‘rough eye’ phenotype, and a certain percentage of the rough-eyed flies possess a variable amount of patches with increased degeneration in these rough eyes<sup>27,28</sup>. Rough eyes containing these degenerative patches (further referred to as degenerated rough eyes) are considered to be more affected compared to the rough eyes without these patches, and this is, therefore, a useful tool to identify enhancers or suppressors of the SCA3-induced toxicity<sup>32</sup>. To visualize these phenotypic differences at a higher magnification and to evaluate the severity of the rough versus degenerated rough eye phenotypes in more detail, we performed correlative light microscopy and scanning electron microscopy on the eyes



**Figure 1. Increased tissue degeneration is present in neurodegenerative patches within the rough eye background of SCA3 flies**  
Eyes of SCA3 flies were visualized in detail using light end electron microscopy. Representative (A, B, C) light microscopy pictures with correlative (A', A'', B', B'', C', C'') scanning electron microscopy pictures of eye phenotypes are shown. A, A', A''. Normal control eye phenotype B, B', B''. SCA3-expressing fly with an eye phenotype classified as rough eyes. C, C', C''. SCA3-expressing fly with an eye phenotype classified as degenerative eyes; black patches show less preservation of tissue integrity.

of wild type flies (Figure 1A-A'') and flies overexpressing human *ATXN3* (Figure 1B-C). It appeared that, the rough eyes consist of irregular formed ommatidia and bristle structures that were despite their irregularity, still clearly visible (Figure 1B-B''). In contrast, the degenerative patches contained an undefined structure and bristles were absent (Figure 1C-C''). These results confirmed that degenerative patches could indeed be classified as more severely affected tissue parts as compared to the rest of the rough eye structures. Therefore, an intervention that causes a decreased amount of rough eyes with degenerative areas within the SCA3 background can be classified as protective.

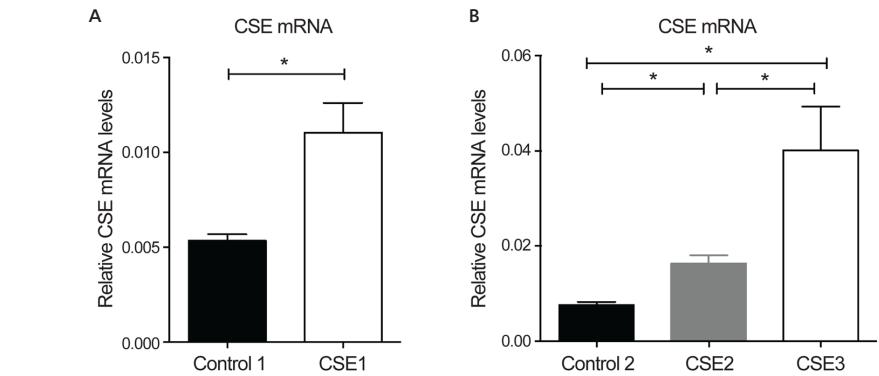
Generation and characterization of various CSE transgenic lines

To further investigate a possible modulating role of CSE in SCA3 pathogenesis, we investigated effects of CSE overexpression in the SCA3 fly model. Six different fly lines overexpressing Eip55E, a highly conserved *Drosophila* ortholog of the human CSE gene (<http://flybase.org/blast>)<sup>33</sup> under a GAL4-inducible promoter, were created. Due to the absence of an antibody against *Drosophila* CSE, the characterization of the lines was performed using qRT-PCR analysis. CSE expression levels of each inducible line were determined in the presence of a daughterless driver resulting in ubiquitous expression of the construct. All six lines showed increased mRNA expression of CSE compared to the in-house control w1118 strain (Supplementary Figure 1A). As the genetic background of *Drosophila* plays an important role in the severity of specific phenotypes<sup>34</sup> all transgenic overexpressing lines were backcrossed for at least 6 generations to create isogenic controls. This resulted in the generation of CSE overexpressing strain 1 (referred to as CSE1) and its specific isogenic control (referred to as control 1) and to the generation of CSE expressing strain 2 and 3 (referred to as CSE2 and CSE3) and their isogenic control (referred to as control 2). This approach allowed us to compare the effect of CSE overexpression in 2 genetic backgrounds, to investigate the effect of variations in overexpression levels and to compare this to isogenic controls. By using qRT-PCR, we demonstrated that CSE1 showed a 2.1-fold induction of CSE compared to its isogenic control, and that CSE2 and CSE3 showed a 2.2-fold and a 5.3-fold increased expression of CSE compared to their isogenic control, respectively (Figure 2A, 2B). Extensive description of all used *Drosophila* genotypes is presented in *Supplementary Materials and Methods* and *Supplementary Figure 1A,B*.

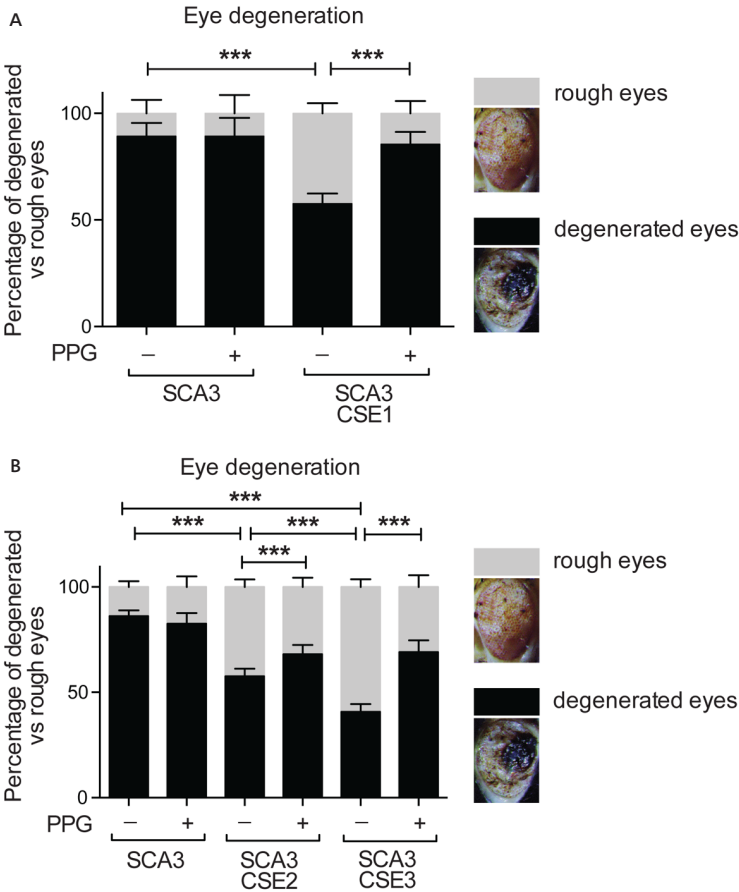
Overexpression of CSE partially rescues the phenotype of SCA3 in *Drosophila*

To investigate a possible effect of CSE overexpression on the eye phenotype, we scored the percentage of degenerated eyes (=when degenerated patches visualized in Figure 1C-C'' are present) one day after

eclosion using light microscopy. In the SCA3 background, CSE-overexpressing flies showed a significant decrease in the percentage of degenerative rough eyes (Figure 3A and 3B). Suppression of the SCA3 degenerative rough eye phenotype was observed in all CSE-overexpressing lines compared to their SCA3 expressing isogenic control lines. Similar results were obtained in both genetic backgrounds. The CSE3 line with the highest level of CSE overexpression reduced the number of degenerative eyes to a greater extent than the CSE2 line (Figure 3B). To further strengthen the rescue potential of CSE, we pharmacologically inhibited CSE with propargylglycine (PPG) as previously described<sup>10</sup>. Supplementation of PPG to the fly food reversed the protective effect of CSE overexpression in the SCA3 background as evidenced by an increased percentage of degenerated rough eyes (Figure 3A and 2B). Addition of PPG



**Figure 2. Expression levels of CSE mRNA in CSE overexpressing flies lines**  
**A and B.** CSE overexpression was determined using qRT-PCR in CSE (CSE1; CSE2; CSE3) overexpressing transgenic fly lines compared to their isogenic controls (control 1 and control 2). CSE was expressed ubiquitously using an actin-GAL4 driver. In both genetic backgrounds, CSE mRNA levels were increased in the CSE overexpressing lines compared to their isogenic controls. \*P<0.05, error bars indicate SEM.



**Figure 3. Overexpression of CSE suppresses SCA3-associated degeneration in *Drosophila***  
**A and B.** SCA3 flies with and without overexpression of CSE (3 independent lines) were analyzed. In all three transgenic lines in the SCA3 background, CSE overexpression resulted in decrease of the degree of eye degeneration compared to isogenic SCA3 expressing lines. Inhibition of CSE by 2mM PPG diminished this effect. The presence of degenerative patches (black area) was determined using light microscopy. For quantification, the number of rough and degenerated eyes in at least three independent experiments (n=100-300 per experiment) was counted. \*\*\*P<0.001, error bars indicate SEM. White area represents the percentage of rough eyes containing neurodegenerative patches. Grey area represents percentage of rough eyes without neurodegenerative patches.



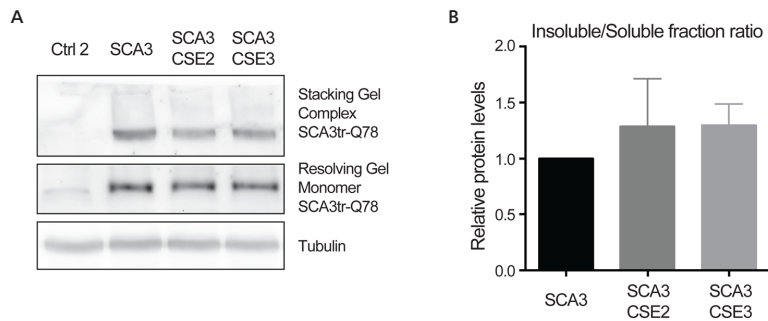
neither enhanced, nor suppressed the percentage of degenerative eyes in the SCA3 background, strongly suggesting that the observed effect in the CSE overexpressing background is due to inhibition of CSE and not due to other effects of PPG. Together, these results indicate that the rescuing potential is mediated by overexpression of CSE and is not influenced by the genetic background.

Overexpression of CSE does not induce a change in levels of insoluble proteins

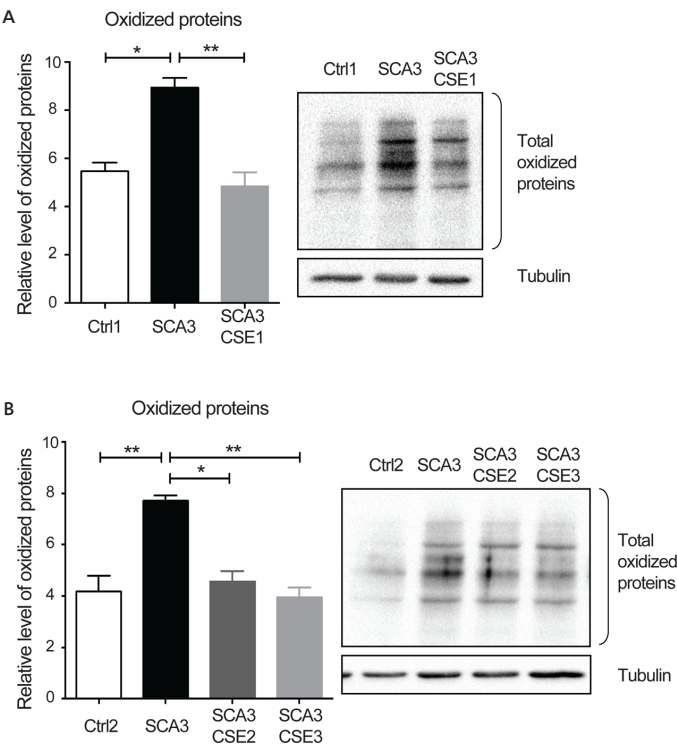
Polyglutamine diseases are thought to be driven by protein aggregation that subsequently triggers a myriad of downstream consequences ultimately leading to neurodegeneration. We thus first tested whether the rescue of SCA3-induced tissue degeneration by CSE overexpression was associated with reduced aggregation of the truncated human ATXN3 protein. Hereto, we determined ratios of insoluble versus soluble fractions of ATXN3 proteins in SCA3 flies in the absence and presence of CSE overexpression using Western blot analysis as described previously<sup>29</sup>. An increased insoluble/soluble ratio indicates an increase in protein aggregation. Overexpression of CSE did not significantly alter the insoluble/soluble ratio in SCA3-expressing flies (Figure 4, Supplementary Figure 2), indicating that the protective effects of CSE overexpression are not mediated by decreased toxic protein aggregates and most likely work protective against damaging effects downstream of the formation of aggregates.

Overexpression of CSE reduces levels of oxidative damage of proteins in SCA3 flies

Oxidative stress is associated with the pathogenesis of SCA3 disease<sup>35, 36</sup>. Previously, it has been demonstrated that CSE deficiency is linked to increased levels of oxidative stress<sup>37</sup>. As a read-out for oxidative stress we used OxyBlot analysis as previously described<sup>38</sup>. SCA3 flies showed increased levels



**Figure 4. SCA3tr-Q78 protein expression and aggregation is not altered in the presence of CSE overexpression**  
**A.** Western blot analysis of extracts of heads of SCA3-overexpressing flies was used to determine levels of protein aggregation. The samples were analyzed for the amount of soluble SCA3tr-78 protein (present in resolving gel) and levels of SCA3tr-78 protein aggregation (present in stacking gel) using an anti-HA antibody. Alpha-tubulin was used as a loading control. In the SCA3 flies, both soluble monomer (in the resolving gel) and aggregated protein (in the stacking gel) fractions were detected. CSE overexpression did not significantly alter the solubility of the SCA3 protein. **B.** Quantification of the ratio between the relative intensity of the protein band in the stacking gel and SCA3tr-78 monomer band in the resolving gel. There was no significant change in the protein solubility upon the overexpression of CSE in the SCA3 background, n=5.

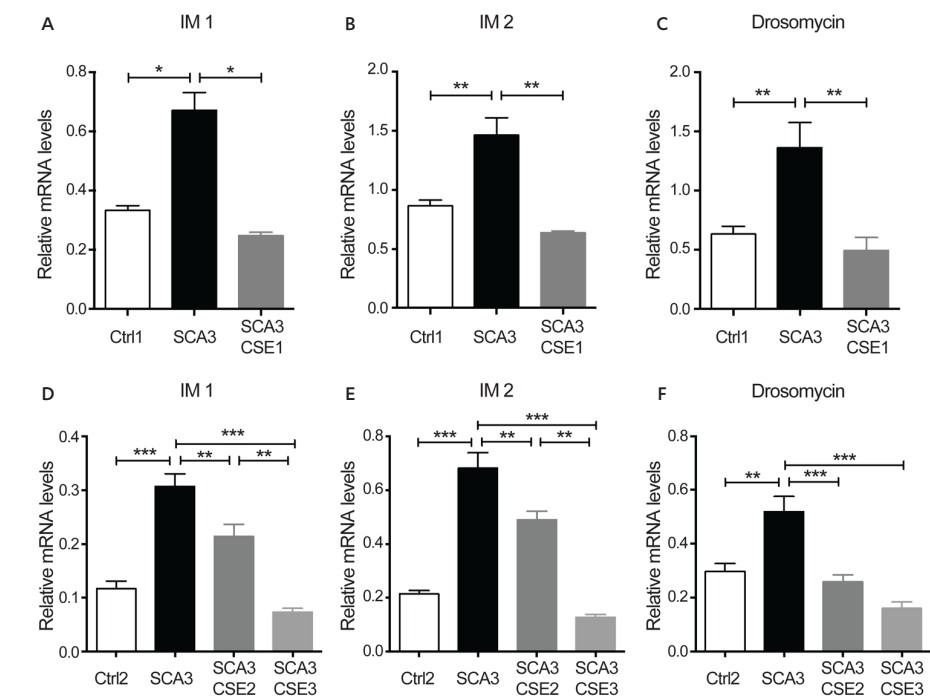


**Figure 5. Increased levels of oxidized proteins in SCA3 flies are rescued by CSE overexpression**  
**A and B.** OxyBlot analysis of extracts derived from fly heads was used to examine levels of oxidized proteins. SCA3 flies had higher total levels of oxidized proteins compared to wild type flies. CSE overexpression in the SCA3 background showed a reduction of oxidized proteins. 3 independent CSE overexpressing lines were used and compared with their isogenic controls. For quantification, optical density of oxidized proteins was normalized to tubulin; levels of oxidized proteins in CSE-overexpressing SCA3 fly samples were comparable to levels in control fly heads. \*P<0.05, \*\*P<0.01, error bars indicate SEM.

of oxidized proteins (characteristically visible as multiple bands) compared with their isogenic non-SCA3 control lines (Figure 5A and 5B). The level of oxidized proteins was reduced in all three CSE overexpression lines in the SCA3 background as compared to the isogenic controls (Figure 5A and 5B).

Overexpression of CSE prevents SCA3-associated immune induction

Several findings suggest that inflammation contributes to the multifaceted pathogenesis of SCA3 disease<sup>39</sup>. In *Drosophila*, the Toll and IMD immune signaling pathways mediate activation of nuclear factor  $\kappa$ B (NF $\kappa$ B) transcription factors Dif-Dorsal and Relish, respectively. Targets of these transcription factors include antimicrobial peptides (AMPs). These immune pathways are equivalent to NF $\kappa$ B signaling in mammals and are a key factor in the induction of the innate immune response<sup>40</sup>. Immune induction by expression of SCA3 in *Drosophila* has been previously reported as well<sup>41</sup>. To investigate whether CSE-mediated protection against SCA3 is associated with a reduction in the immune response, we performed

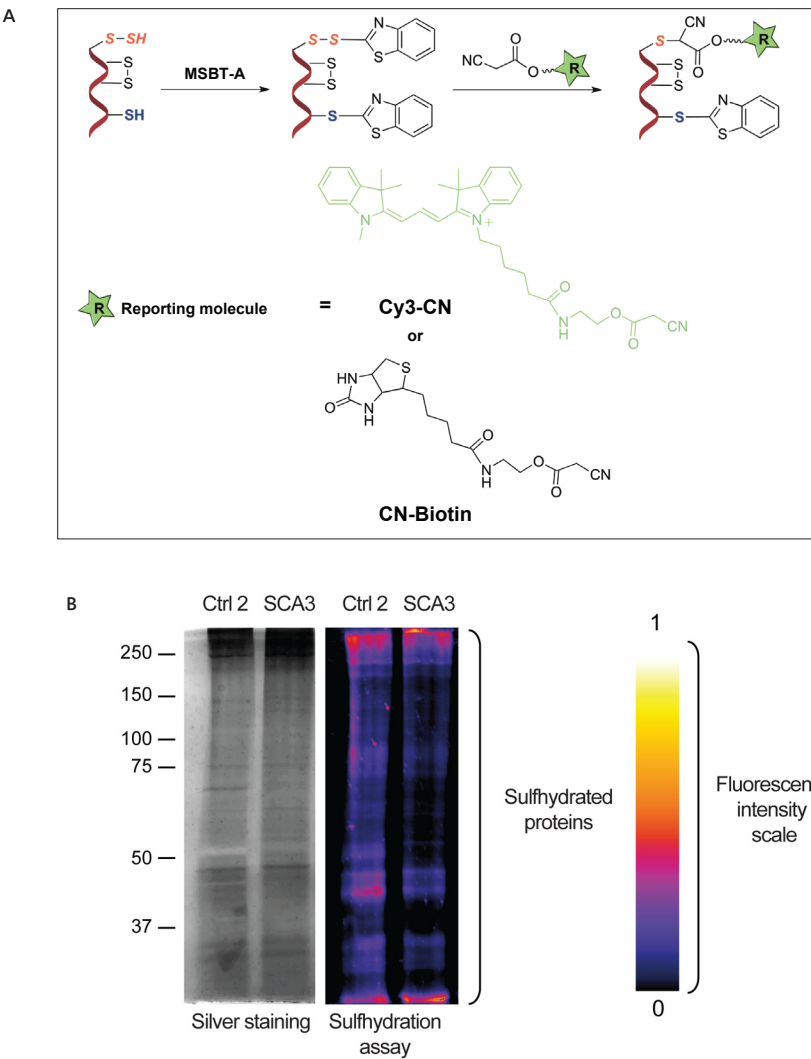


**Figure 6. Overexpression of CSE prevents SCA3-related immune induction**  
mRNA levels of various immune response genes (IM1, IM2, Drosomycin) were determined by qRT-PCR in control flies, in SCA3 flies (2 genetic backgrounds) and in SCA3 flies overexpressing CSE in the same isogenetic background. In SCA3 expressing flies all investigated immune players were upregulated compared to control flies of the same genetic background. In CSE expressing flies in various SCA3 backgrounds, immune response gene expression was lower compared to the isogenetic SCA3 backgrounds. \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001, error bars indicate SEM. IM 1 = immune-induced molecule 1, IM 2 immune-induced molecule 2

a qRT-PCR analysis for AMPs, which are targets of either the IMD or the Toll pathway. As targets of the Toll pathway, *immune-induced molecule 1* (IM1), *immune-induced molecule 2* (IM2) and *drosomycin* were analyzed<sup>42, 43</sup> for the IMD pathway, *attacin*, *cecropin A1* and *diptericin* were analyzed<sup>40</sup>. Expression of SCA3 activated the immune response of both pathways. CSE overexpression in the SCA3 background significantly attenuated all investigated players of the Toll pathway (Figure 6). A comparable effect was seen for a subset of AMPs of the IMD pathway (Supplementary Figure 3). This shows that the suppressive effect of CSE overexpression on eye degeneration in SCA3 flies is associated with a dampening of the Toll pathway and a partial dampening of the IMD innate immune response pathway. This data suggest that CSE may exert its beneficial effects on the SCA3 phenotype by attenuating the immune response.

SCA3 flies show reduced levels of protein persulfidation

Decreased levels of CSE are associated with impaired neurological function<sup>22</sup>. Here we demonstrated that increased levels of CSE are protective. Because CSE overexpression is associated with increased protein



**Figure 7. Protein persulfidation is decreased in SCA3 flies**  
**A.** Schematic overview of the tag-switch assay and the structures of the probes that introduce either biotin labeling (CN-biotin) or fluorescence labeling (CN-Cy3) as reporter molecules. **B.** Persulfidation levels in the SCA3 fly heads are decreased compared to control flies. Protein persulfidation was determined using the tag-switch assay with direct fluorescence labeling and in-gel fluorescence detection. The gels were artificially colorized in ImageJ for better visualization of the changes in the signal intensity. Fluorescence intensity scale is provided at the right. Silver-stained gels were shown to demonstrate equal protein loading of the samples. Note that due to the sensitivity of the methods not all bands visualized by silver staining will be detected by the fluorescence detection of protein persulfidation and vice versa. Fluorescence intensity scale is provided; signal in the white-yellow range of colors indicate relatively high levels of protein persulfidation; signal in the black-blue range indicate relatively low levels of protein persulfidation. Extracts of control flies and SCA3 flies were loaded. MSBT-A= a water-soluble methylsulfonyl benzothiazole derivate.



persulfidation<sup>24,44</sup>, we hypothesized that in a SCA3 background protein persulfidation may be decreased and that CSE overexpression may lead to restoration of this posttranslational modification.

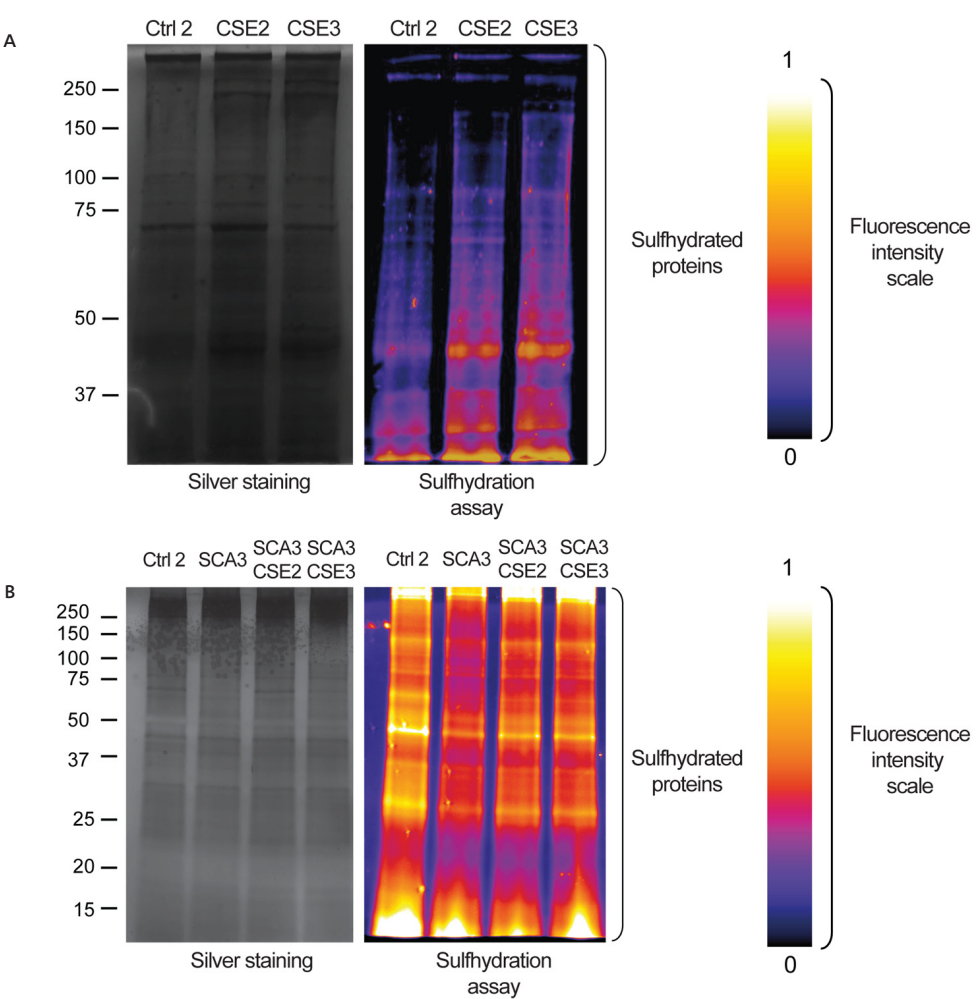
To selectively detect protein persulfidation, a recently reported tag-switch assay was further optimized and used (Figure 7A). The original method labels the persulfidation of proteins with biotin as a reporting tag (Supplementary Figure 4A-C). To further enhance the signal intensity, the tag-switch assay was improved by using a reagent, which directly introduced a cyanine-based Cy3 fluorescence tag, **Cy3-CN** (Figure 7A) to persulfidated proteins. An artificial color scheme was used to better illustrate the persulfidation levels (the color scale is given in the figure). Signal in the yellow-white range indicates high levels of persulfidation and signal in the black-blue range indicates a low signal (see also supplementary information). Persulfidation of proteins in heads of SCA3 flies was indeed decreased in comparison with wild type controls (Figure 7B). Next, we investigated whether the protective effect of CSE overexpression was associated with restoration of protein persulfidation. Consistently with our hypothesis, we found that CSE overexpressing flies demonstrated elevated protein persulfidation as compared to the controls (Figure 8A, Supplementary Figure 4C). The observed rescue of SCA3 degeneration in CSE overexpressing fly lines was associated with normalization of protein persulfidation levels (Figure 8B, Supplementary Figure 4A and 4B). These data showed that degenerative defects of SCA3 are associated with decreased levels of protein persulfidation and this can be reversed by overexpression of CSE, an intervention that also protects against tissue loss in the *Drosophila* SCA3 model.

Treatment with sodium thiosulfate reduces eye degeneration in SCA3 flies

To try to pharmacologically rescue SCA3-induced degeneration we used sodium thiosulfate (STS). STS is a relatively stable non-toxic compound used in clinical settings to treat caliciphylaxis, extravasations during chemotherapy or cyanide poisoning<sup>45</sup>, and is also a component of the transsulfuration pathway. Used as a substrate for rhodanase-like enzymes, thiosulfate could also be a source of targeted persulfidation, as recently proposed by Mishanina et al.<sup>31</sup>. Therefore, we tested the effects of thiosulfate on SCA3 flies. Increasing concentrations of STS were fed to SCA3-expressing flies to investigate possible toxicity of STS. Concentrations of 120mM STS and higher induced lethality (data not shown). Upon supplementation of lower concentrations of STS, a dose-dependent decrease in the percentage of degenerated rough eyes was observed (Figure 9A). 80mM STS reduced the percentage of degenerated rough eyes in SCA3-overexpressing conditions in two genetic backgrounds (Figure 9B). These data show that the suppression of SCA3-associated degeneration is achieved not only using a genetic approach by overexpression of CSE but can also be achieved pharmacologically using STS.

Endogenous CSE is present in affected brain tissue of SCA3 patients

In order to investigate a possible role of CSE in human SCA3 pathogenesis, we investigated the expression levels and localization of CSE in healthy tissue and in SCA3 disease tissue. To determine the presence and localization of CSE, we performed immunohistochemistry for CSE on post-mortem pontine tissue of control individuals and of SCA3 patients. From the sparse tissue available for this rare disease, pontine tissue was chosen to analyze the features of this disorder, because in this tissue several types



**Figure 8. Overexpression of CSE increases protein persulfidation in the wild type background and partly rescues decreased protein persulfidation of SCA3 flies**

**A.** CSE overexpression in wild type flies elevates levels of persulfidation. Protein persulfidation was determined using the tag-switch assay with direct fluorescence labeling and in-gel fluorescence detection. Extracts of flies overexpressing CSE showed an increase of protein persulfidation compared to the control flies. The gels were artificially stained in ImageJ for better visualization of the changes in the signal intensity. Fluorescence intensity scale is provided at the right. Silver-stained gels were shown to demonstrate equal protein loading of the samples. Note that due to the sensitivity of the methods not all bands visualized by silver staining will be detected by the fluorescence detection of protein persulfidation and vice versa. Fluorescence intensity scale is provided; signal in the white-yellow range of colors indicate relatively high levels of protein persulfidation; signal in the black-blue range indicate relatively low levels of protein persulfidation. **B.** Protein persulfidation levels in the SCA3 fly heads are decreased compared to control flies and CSE overexpression in the SCA3 background resulted in the partial restoration of persulfidation levels. MSBT-A= a water-soluble methylsulfonyl benzothiazole derivate.

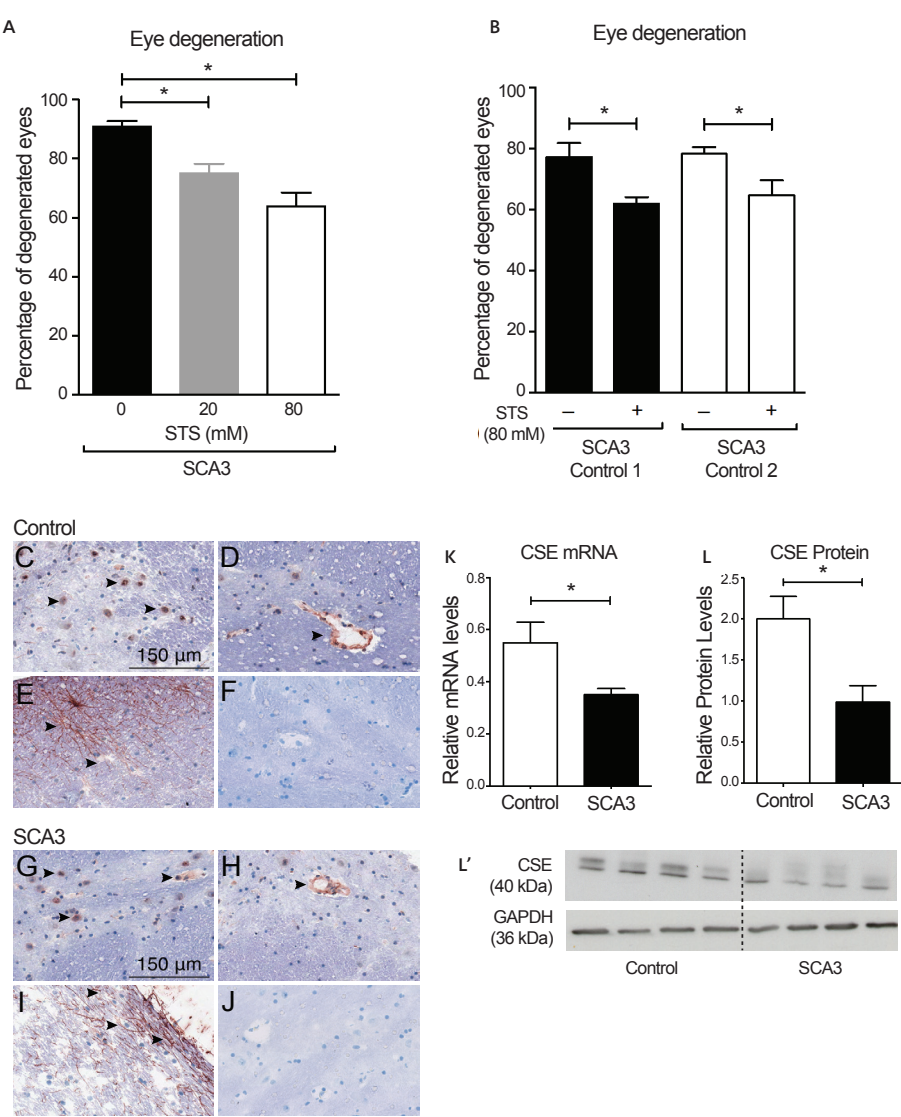
of toxic protein aggregates are present with enough neurons preserved to allow immunohistochemical analysis<sup>7</sup> in contrast to other brain areas that are almost completely degenerated or that hardly show degeneration or protein aggregation<sup>46, 47</sup>. As control samples, post-mortem tissue of individuals without a neurodegenerative or neuropsychiatric disease were used (Supplementary Table 1, n=7). CSE protein expression was observed in vascular endothelium, neurons and astrocytes (Figure 9C-J). This localization pattern was not affected in pontine tissue of SCA3 patients (Figure 9C-J; Supplementary Figure 5). To investigate expression levels of CSE, we performed qRT-PCR analysis for CSE transcripts on pontine samples of SCA3 patients and control samples. qRT-PCR data revealed the presence mRNA levels of CSE in pontine tissue of control tissue (n=7) and SCA3 (n=6) patients, although in the latter levels were reduced (Figure 9K). Western Blot analysis using an anti-CSE antibody<sup>48</sup> further confirmed the presence and decreased levels of endogenous CSE in pontine tissue of SCA3 patients compared to controls (Figure 9L, L'). Together these data demonstrated that CSE is endogenously present but decreased expressed in affected brain areas of SCA3 patients.

## DISCUSSION

We present evidence that CSE overexpression works protective in a *Drosophila* model for the neurodegenerative disease SCA3. To our knowledge, protective effects by CSE overexpression in neurodegenerative animal models have not been described before. However, neuroprotective effects of H<sub>2</sub>S have been reported previously not only in experimental models for Parkinson's disease<sup>21</sup>, vascular dementia<sup>49</sup> and homocysteine-induced neurotoxicity, but also in *in vitro* models for oxidative stress in neurons<sup>50</sup> and Alzheimer's disease<sup>51</sup>. In an experimental mouse model for Parkinson's disease, inhalation of H<sub>2</sub>S prevents the development of neurodegeneration and movements disorders<sup>20</sup>.

The findings in *Drosophila* may be of clinical relevance because we observed that in SCA3 patients CSE expression is decreased in affected brain areas compared to controls. Recently, decreased levels of CSE were also demonstrated in striatal brain samples from patients with Huntington's disease<sup>22</sup>. It was also shown that CSE<sup>-/-</sup> mice show impaired locomotor functions<sup>22</sup>, therefore, it is possible that low levels of CSE negatively influence the progression of neurodegenerative phenotypes in Huntington's disease and in SCA3. This is consistent with our findings showing that, in contrast to decreasing CSE levels, boosting CSE expression in a neurodegenerative background is beneficial. In contrast to our results, CSE protein levels were found unaltered in the cerebellum and cerebral cortex of one spinocerebellar ataxia patient (SCA subtype unknown)<sup>22</sup>, suggesting that alteration of CSE levels in SCA patients may be confined to pontine tissue or may depend on the SCA subtype.

CSE is an essential enzyme in the transsulfuration pathway and plays a role in the endogenous production of cysteine<sup>52</sup> and H<sub>2</sub>S<sup>53-55</sup>. Therefore, the beneficial effects of CSE can be mediated via cysteine, H<sub>2</sub>S or both. It is also possible that the increased expression of CSE catalyzes the formation of cysteine persulfides that can trans-persulfidate the proteins without any H<sub>2</sub>S being produced<sup>56</sup>. The explanation of the beneficial effects by protein persulfidation is in agreement with our observations that this posttranslational



**Figure 9. Treatment with STS suppresses SCA3-associated degeneration in *Drosophila*, and CSE levels are decreased in brains of SCA3 patients**

**A and B.** Effect of the H<sub>2</sub>S donor sodium thiosulfate was determined on the degenerative eye phenotype by using light microscopy. The number of rough and degenerated eyes was counted in three independent experiments (n=100-300 per experiment). **A.** Increasing concentrations of STS resulted in a reduced percentage of degenerated rough eyes of SCA3-expressing flies in the control background. **B.** Addition of 80mM STS to the food of SCA3-expressing lines in two genetic backgrounds partly rescued the SCA3-induced eye degenerative phenotype. **C-J.** Immunohistochemistry by using an anti-human CSE antibody revealed that in control human pontine tissue (**C-F**) and in pontine tissue of SCA3 patients (sample 5, Supplementary Table S1) (representative images are shown in **G-J**), CSE is localized in neurons of the pontine nuclei (**C, G**), the vasculature (**D, H**) and astrocytes (**E, I**). Black arrows indicate the mentioned structures. No differences in staining pattern were observed between control and SCA3 brain tissue. Omission of the primary antibody resulted in absence of staining; representative images are shown (**F, J**). Scale bar indicates 150μm in all images. **K.** CSE mRNA levels were determined by using qRT-PCR (control, n=7; SCA3, n=6). **L, L'.** CSE protein levels were determined using Western blot analysis. Control samples correspond with control: 2, 3, 4, 6, respectively (Supplementary Table S1); SCA3 samples correspond with SCA3: 2, 3, 4, 5, respectively (Supplementary Table S1). \*P < 0.05, error bars indicate SEM.

modification is increased in CSE overexpressing flies and restored in CSE overexpressing flies in a SCA3 background. Moreover, it also explains the rescue effect of STS, assuming that the proposed effect of STS on protein persulfidation is correct<sup>31</sup>. A protective effect of protein persulfidation has been demonstrated in other studies as well. For example, the activity of neuroprotective ubiquitin ligase parkin is regulated by protein persulfidation. Parkin persulfidation is markedly depleted in the brains of patients with Parkinson's disease<sup>26</sup>. Another study demonstrates that the capacity of H<sub>2</sub>S to protect against oxidative stress is executed via persulfidation<sup>24, 25</sup>. These findings together with our results suggest that boosting of the transsulfuration pathway may contribute to neuroprotection via increased persulfidation of proteins.

Our results show that overexpression of CSE is associated with a dampening of the immune response and decreased levels of protein oxidation. This is consistent with previous findings because inflammation has been implicated as a critical mechanism responsible for the progressive nature of neurodegeneration<sup>39, 57</sup> and there is an inverse link between an activated transsulfuration pathway and the immune response. In experimental models, H<sub>2</sub>S exerts anti-neuroinflammatory effects via inhibition of p38/Jun nuclear kinase and NFκB signaling pathways<sup>58</sup>, and the inhibition of CSE by PPG leads to increased inflammation<sup>59</sup>. Furthermore, CSE has been shown to be a modulator of oxidative stress in mice<sup>37</sup>. SCA3 is associated with oxidative stress because mutant ATXN3 is associated with a significantly reduced capability to counteract oxidative stress that contributes to neuronal cell death in SCA3<sup>60</sup>.

Based on the discussed results of others and our observations, we propose the following hypothetical model: polyQ diseases lead to accumulation of toxic protein aggregates, and this somehow reduces levels of CSE and/or protein persulfidation. This, in turn, contributes to increased oxidative stress and an augmented immune response leading to accelerated neurodegeneration. It is possible that overexpression of CSE induces protein persulfidation (via or independent from induced H<sub>2</sub>S and/or cysteine biosynthesis). Increased levels of protein persulfidation reduces the levels of oxidative stress, dampens the immune response and, by this, the damaging effects of toxic protein aggregates are reduced and tissue integrity is better preserved.

Our data show that CSE levels are decreased in tissue of SCA3 patients. However, to our opinion, it is of more importance that CSE is still expressed in affected tissue and apparently in identical cell types, because this allows a strategy to increase CSE expression or activity by pharmacological inventions to protect against tissue degeneration in SCA3. Little is known about the regulation of CSE, but there are substances available that are able to influence CSE activity or transcription. There is evidence that myeloid zinc finger 1 and specificity protein 1 transcription factor affect the transcription of CSE<sup>61</sup>. Furthermore, studies suggest that CSE can be upregulated by bacterial endotoxin<sup>61, 62</sup> and by nitric oxide<sup>63</sup>. S-adenosylmethionine and pyridoxal-5'-phosphate stimulate CSE activity to increase H<sub>2</sub>S production<sup>64, 65</sup>. Alternatively to increasing CSE expression as a therapeutic option, rescue may be provoked by STS because our data show a protective effect of this compound as well and it is tolerated by humans in high concentrations<sup>66, 67</sup>.

Our data indicate a modifying role of the transsulfuration pathway in SCA3 and suggest that this is mediated via protein persulfidation. The presence of CSE in SCA3-relevant brain regions, together with

the protective effects of CSE overexpression in *Drosophila*, indicates the relevance for future research on developing clinically applicable activators of CSE or other members of the transsulfuration pathway. As the protective effects occur downstream of the formation of protein aggregates, it may be possible that activation of the transsulfuration pathway is protective for other polyglutamine expansion-induced diseases as well.

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# SUPPLEMENTARY MATERIALS AND METHODS

## Drosophila stocks

For overexpression of the genes, the UAS-GAL4 binary system for targeted gene expression was used<sup>1</sup>. Eip55E (*Drosophila* CSE) cDNA was cloned into the pUAST vector and verified by sequencing. Transgenic CSE fly lines were generated by Genetic Services Inc. (Sudbury, USA) in a w<sup>1118</sup> (y<sup>w</sup>1118) background by random insertion into the genome. Fly stocks bearing the CSE transgene were backcrossed into control lines for six generations. CSE-1A was isogenized with the w<sup>1118</sup>-A line; and CSE-2B and CSE-3B were isogenized with the w<sup>1118</sup>-B line. Transgenes were selectively overexpressed in the eyes by using GMR-GAL4 driver flies (stock #1104), ubiquitously overexpressed using the daughterless driver flies (stock #8641) for the qPCR analysis or overexpressed using the actin driver (stock #4414) for the protein persulfidation measurements. To test whether a titration effect existed, a UAS-GFP (stock #6658) and two UAS-YFP (stock #6659; #6660) lines were used. The driver stocks and stocks bearing fluorescent proteins under the control of UAS were ordered from Bloomington *Drosophila* stock center (Indiana University, USA). All crosses were performed at 25°C according to standard protocols. For all the experiments only male flies were used.

## Genotypes of fly lines used per experiment

Experiment	Genotype
qRT-PCR analysis of CSE-overexpressing lines (Figure 2, Supplementary Figure S1)	y <sup>1</sup> , w <sup>1118</sup> -1; daughterless-GAL4/+
	y <sup>1</sup> , w <sup>1118</sup> -1; daughterless-GAL4/+; UAS-CSE-1/+
	y <sup>1</sup> , w <sup>1118</sup> -2; daughterless-GAL4GAL4/+
	y <sup>1</sup> , w <sup>1118</sup> -2; daughterless-GAL4/+; UAS-CSE-2/+
	y <sup>1</sup> , w <sup>1118</sup> -2; daughterless-GAL4/UAS-CSE-3
Co-overexpression of CSE and SCA3 in <i>Drosophila</i> eyes (Figure 1, 3)	y <sup>1</sup> , w <sup>1118</sup> -2
qRT-PCR analysis of antimicrobial peptide mRNA abundance (Figure 6, Supplementary Figure 3)	y <sup>1</sup> , w <sup>1118</sup> -2; GMR-GAL4-UAS-SCA3trQ78/+
Oxidative stress assay (Figure 5)	y <sup>1</sup> , w <sup>1118</sup> -2; GMR-GAL4-UAS-SCA3trQ78/+; UAS-CSE-1/+
Western Blot analysis of SCA3trQ78 aggregation (Figure 4, Supplementary Figure 2)	y <sup>1</sup> , w <sup>1118</sup> -2
Protein persulfidation analysis (Figure 8B, Supplementary Figure 4A,B)	y <sup>1</sup> , w <sup>1118</sup> -2; GMR-GAL4-UAS-SCA3trQ78/+
	y <sup>1</sup> , w <sup>1118</sup> -2; GMR-GAL4-UAS-SCA3trQ78/UAS-CSE-3
The effect of thiosulfate supplementation on the eye phenotype of SCA3 flies (Figure 9)	y <sup>1</sup> , w <sup>1118</sup> -1; GMR-GAL4-UAS-SCA3trQ78/+
	y <sup>1</sup> , w <sup>1118</sup> -2; GMR-GAL4-UAS-SCA3trQ78/+
Protein persulfidation analysis (Figure 7B, Figure 8A, Supplementary Figure 4C)	y <sup>1</sup> , w <sup>1118</sup> -1; actin-GAL4/+
	y <sup>1</sup> , w <sup>1118</sup> -1; actin-GAL4/+; UAS-CSE-1/+
	y <sup>1</sup> , w <sup>1118</sup> -2; actin-GAL4/+
	y <sup>1</sup> , w <sup>1118</sup> -2; actin-GAL4/+; UAS-CSE-2/+
	y <sup>1</sup> , w <sup>1118</sup> -2; actin-GAL4/UAS-CSE-3

Fly food and supplementation of PPG and STS

Fly strains were raised and crossed on Nutri-Fly Bloomington food (Brewer’s Yeast, Sucrose, Agar Type II, Glucose, Yeast Extract, MgSO<sub>4</sub> x 6H<sub>2</sub>O, Peptone, CaCl<sub>2</sub> x 2H<sub>2</sub>O; Genesee Scientific). For inhibition of CSE, fly crosses were set up on propargylglycine (PPG)- supplemented food. PPG (Sigma, Zwijndrecht, the Netherlands) was added to freshly prepared fly food to a final concentration of 2mM as previously described<sup>2</sup>. In the same manner, STS (Sigma, Zwijndrecht, the Netherlands) was administered to the fly food reaching final concentrations of 20mM, 80mM and 120mM. Every two days, the relevant concentration of STS dissolved in distilled water was added to the vials during development of the flies.

Light and electron microscopy

To enable correlative analysis, the same fly for each condition was used in both light microscopy (LM) and scanning electron microscopy (SEM). One-day-old flies were decapitated, heads were dehydrated through ethanol series, and after acetone as an intermediate step, air dried from tetramethylsilane (Sigma-Aldrich). Light microscopy images of fly eyes were taken with a Leica M165 FC stereomicroscope followed by focus stacking using Adobe Photoshop. For the scanning electron microscopy, the same eyes were gold/paladium-coated (3nm) and analyzed with a Zeiss Supra 55 SEM at 2KV using the SE2 detector.

Quantitative RT-PCR

Human pontine tissue was homogenized in lysis buffer and total RNA was extracted using the RNeasy Mini Kit (Qiagen). To verify CSE overexpression in transgenic *Drosophila* lines under control of daughterless driver, total RNA was isolated from 10-15 one-day old flies using RNeasy Mini Kit (Qiagen). For each genotype/treatment, at least three independent extractions were prepared. cDNA was synthesized using Superscript II with random hexamer primers (Invitrogen, Carlsbad, USA). Gene expression was determined by quantitative real time-PCR (qPCR) using a SYBR green mastermix (iQ SYBR GREEN Supermix; Bio-Rad). Rp49 and TBP were used as normalization reference housekeeping genes for *Drosophila* and human pontine tissue, respectively. The PCR profile consisted of 15 min at 95°C, followed by 40 cycles with heating of 95°C for 15 s and cooling to 60°C for 1 min. To detect mRNA levels of immune response genes, the same procedure was followed with the exception that ten-day-old flies co-overexpressing CSE and SCA3 under control of GMR driver were used. All samples were normalized to their housekeeping gene, the average Ct values for target genes were subtracted from the average housekeeping gene Ct values to yield the delta Ct. Results were expressed as 2<sup>-ΔCt</sup>.

The following primers were used:

Gene	Primer
<i>Drosophila</i> CSE (Eip55E)	For - TGAACGGTCATACGGATGTG Rev - ACCTGATAGCAGTCGAATGG
<i>Drosophila</i> rp49	For - GCACCAAGCACTTCATCC Rev - CGATCTCGCCGCACTAAA
<i>Drosophila</i> IM1	For - TGCCCAGTGCACCTCAGTATC Rev - GATCACATTTCTGGATCGG
<i>Drosophila</i> IM2	For - AAATACTGCAATGTGCACGG Rev - ATCGTGCTTTGGATTGAGG
<i>Drosophila</i> Drosomyacin	For - GTACTTGTTCCGCTCTTCG Rev - GATTTAGCATCCTTCGCACC
<i>Drosophila</i> Diptericin	For - ACCGCAGTACCCACTCAATC Rev - ACTTTCAGCTCGGTCTGA
<i>Drosophila</i> Attacin	For - GCTTCGAAAATAAACTGG Rev - TCCCGTGAGATCCAAGGTAG
<i>Drosophila</i> Cecropin	For - GAACTTCTACAACATCTTCGT Rev - TCCCAGTCCCTGGATTGT
Human CSE	Assay-on-Demand Applied Biosystems ID: Hs00542284_m1
Human TBP	For - GCCCGAAACGCCGAATAT Rev - CCGTGGTTCGTGGCTCTCT

Western blot analysis

For the SCA3tr78 insoluble/soluble fraction analysis, one-day-old flies were directly frozen in liquid nitrogen and decapitated. 25 heads per condition were homogenized in Laemmli Sample Buffer (62,5mM Tris/HCL pH 6,8; 2% SDS; 10% glycerol; bromophenol blue) containing 2% beta-mercaptoethanol. 10 ul of each sample were loaded onto 12.5% SDS-polyacrylamide gels. Proteins were transferred from the gels onto nitrocellulose membranes and immunostained. Aspecific binding of the antibodies was prevented using 5% milk in Tris-buffered saline-0.1% Tween-20 (TBST). Membranes were incubated with the primary antibody solution in 5% milk in TBST overnight at 4 °C. As a primary antibody, rat monoclonal high affinity anti-HA-peroxidase (1:500) (clone 3F10; Roche, Indianapolis, USA) was used to detect SCA3tr-78, and mouse anti-tubulin (Sigma, Zwijndrecht, the Netherlands) was used for tubulin detection. Afterwards, membranes were incubated with the secondary antibody in TBST. Goat anti-rat IgG horseradish peroxidase (1:5000) (GE Healthcare UK Limited) was used as the secondary antibody for SCA3tr-78 detection. As the secondary antibody for alpha-tubulin detection, sheep anti-mouse IgG horseradish peroxidase (1:4000) (GE Healthcare UK Limited) was used. The blot was scanned with an imaging densitometer, and optical densities were quantified. To calculate the ratio between the SCA3tr-78 insoluble and soluble fraction, the total intensity of the signal in the stacking gel normalized to alpha-tubulin was divided by the intensity of SCA3tr-78 monomer band normalized to alpha-tubulin. Western blots from five independent experiments with unique sets of samples were used for quantification using ImageJ.



### Protein oxidation detection

Ten-days-old flies were directly frozen in liquid nitrogen and decapitated. Per condition, 15 heads were homogenized in RIPA buffer containing 2% beta-mercaptoethanol. Protein oxidation was assessed with OxyBlot Protein Oxidation Detection Kit (Millipore, Billerica, USA) according to manufacturer's instructions. In this method, carbonyl groups of the proteins are derivatized using 2,4-dinitrophenylhydrazine (DNPH) reagent, the sample is then processed on the SDS-PAGE in a similar fashion as in the Western blot analysis and, as a result, the total levels of oxidized proteins in the sample are detected<sup>3</sup>. As a loading control, blots were immunostained with a rabbit alpha-tubulin (Sigma, Zwijndrecht, the Netherlands) antibody. The relative levels of oxidized proteins were measured by comparing total chemiluminescence of the sample lanes normalized to alpha-tubulin levels using ImageJ.

### Collection of human pontine tissue

Brains from 7 genetically confirmed SCA3 patients and 7 controls without medical histories of neuropsychiatric diseases were analyzed (table S1). Immediately after brain autopsy samples (15x15x5mm) of the base of the pons were snap frozen. Informed consent was obtained from all SCA3 patients. Control cases were anonymized and coded according to the National Code for Good Use of Patient Material. All procedures were approved of and in accordance with the Medical Ethical Committee of the University Medical Center Groningen.

### Immunohistochemistry for CSE

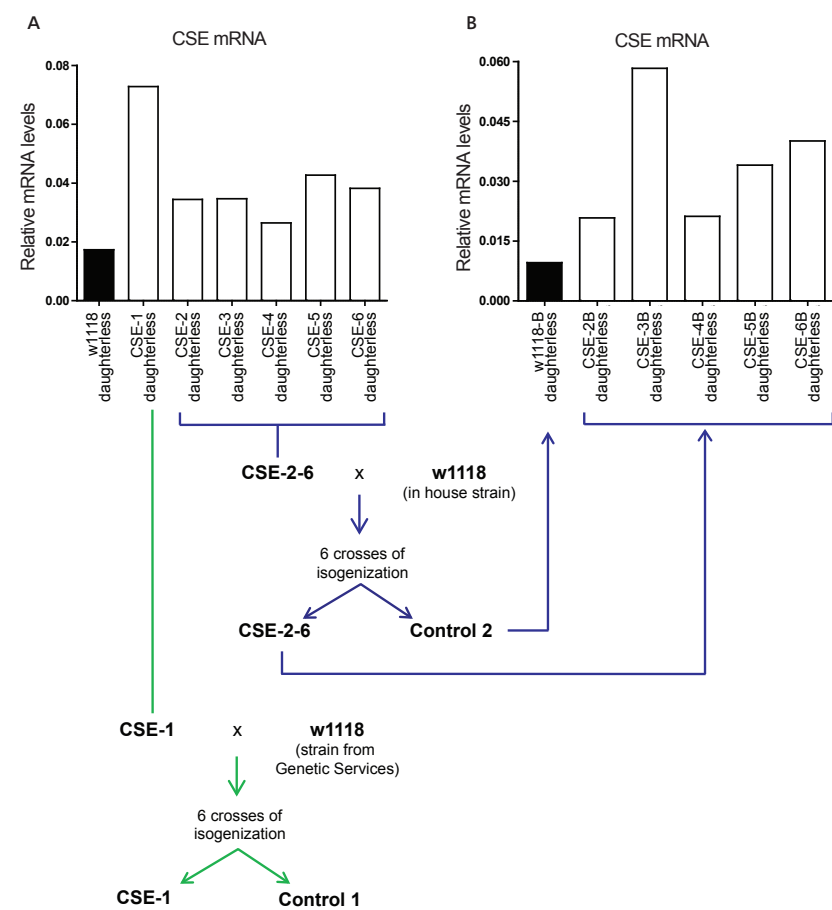
For immunostaining, frozen human pontine sections were dried and fixed in acetone. Subsequently, sections were incubated at room temperature with the primary antibody (Proteintech rabbit polyclonal CSE antibody 11217-1-AP (1:500)) for 60 minutes. Endogenous peroxidase was blocked with H<sub>2</sub>O<sub>2</sub> in phosphate buffered saline (PBS, pH 7.4) for 30 minutes. Binding was detected using sequential incubation with a peroxidase-labeled secondary antibody (Dakopatts, Glostrup, Denmark) for 30 minutes. All antibodies were diluted with PBS supplemented with 1% BSA. At the secondary antibody dilution, 1% human AB serum was added. Peroxidase activity was developed using filtered 3-amino-9-ethylcarbazole for 15 minutes containing H<sub>2</sub>O<sub>2</sub>. Counterstaining was performed using Mayer's hematoxylin. Appropriate isotype and PBS controls were consistently negative.

### Statistical analysis

Data were analyzed using GraphPad Prism 5.0 and IBM SPSS 20.0 software. Normality was tested using the Kolmogorov-Smirnov test. The unpaired Student's t-test was used for comparisons between 2 groups with normal distribution. Non-parametric data were compared using the Mann-Whitney U-test. For comparisons between three groups, an ANOVA with Bonferroni post-test was used for parametric data and a Kruskal Wallis with Dunnett's post-test was used for non-parametric data. For the analysis of the number of degenerated eyes, a logistic regression was used. The number of replications of each experiment was at least three. Statistical significance was accepted at p<0.05. All data are expressed as the mean ± standard error of the mean (SEM) unless indicated otherwise.

## SUPPLEMENTARY REFERENCES

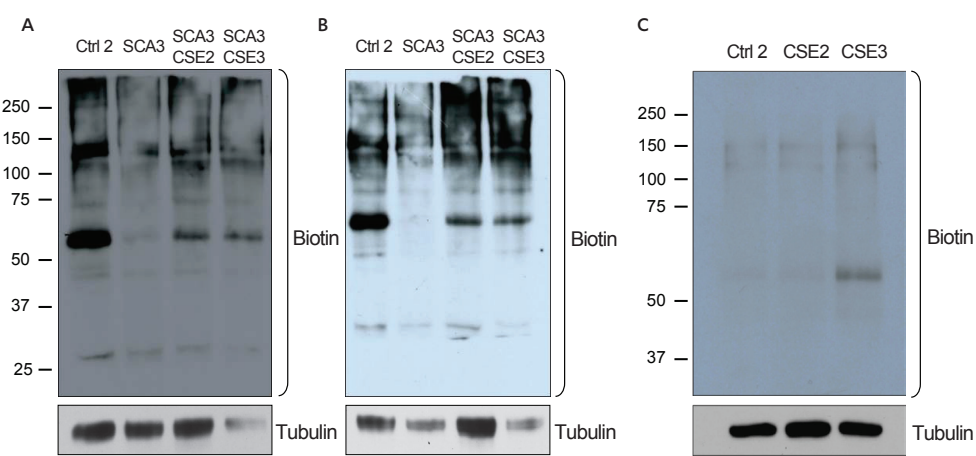
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2. Kabil, H., Kabil, O., Banerjee, R., Harshman, L. G. & Pletcher, S. D. Increased transsulfuration mediates longevity and dietary restriction in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America* **108**, 16831-16836 (2011).
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**Supplementary Figure 1. CSE mRNA levels of different CSE overexpression lines and schematic representation of backcrossing of relevant CSE lines**

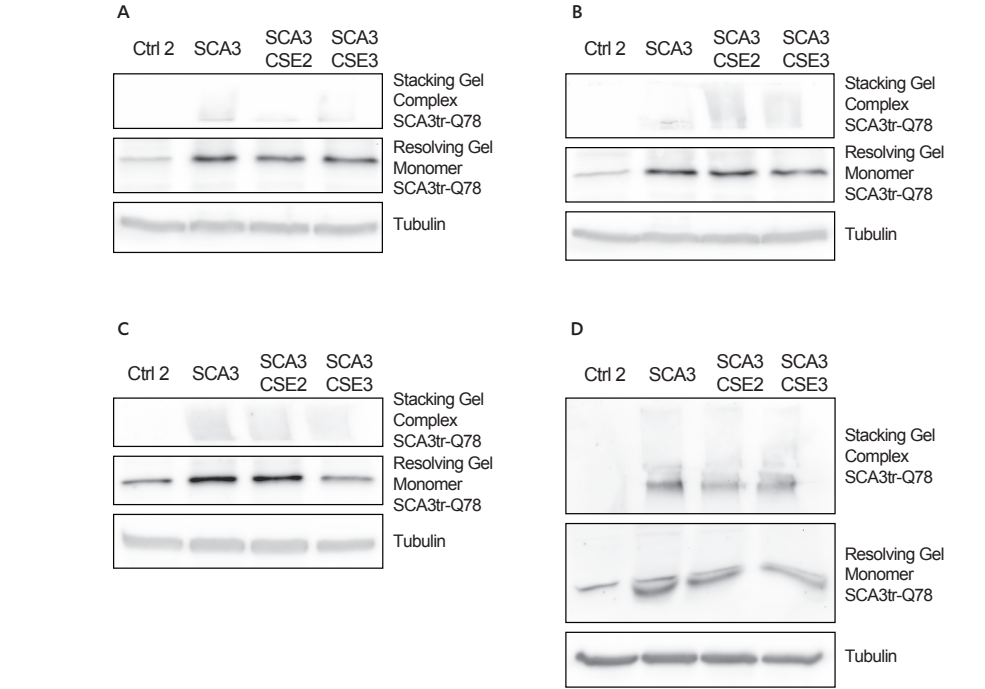
**A.** Relative CSE mRNA levels in fly lines received from Genetic Services and **(B)** in lines after backcrossing for 6 generations into the in-house w1118 strain expressed under control of the daughterless driver. Below: scheme of crosses to isogenize fly lines with 2 different w1118 control strains. Both experiments were used as an indication to select fly lines for further experiments and, therefore, were performed once.

Genetic crosses to generate isogenic lines: As an isogenic control, one strain (strain CSE-1, Supplementary Figure 1) was backcrossed with the w1118 control strain that was used to generate the transgenic lines. The backcrossed CSE strain 1 overexpressing line is further referred to as CSE1, and its isogenic non-CSE-expressing control line is further referred to as control 1. Other lines (CSE-2 to CSE-6) were backcrossed with the in-house w1118 strain to generate an isogenic control line. From these, two lines were selected: one line overexpressing CSE to a lower level (further referred to as CSE2) and one line overexpressing CSE to a higher level (further referred to as CSE3) (Supplementary Figure 1). The isogenic control of these lines is further referred to as control 2.

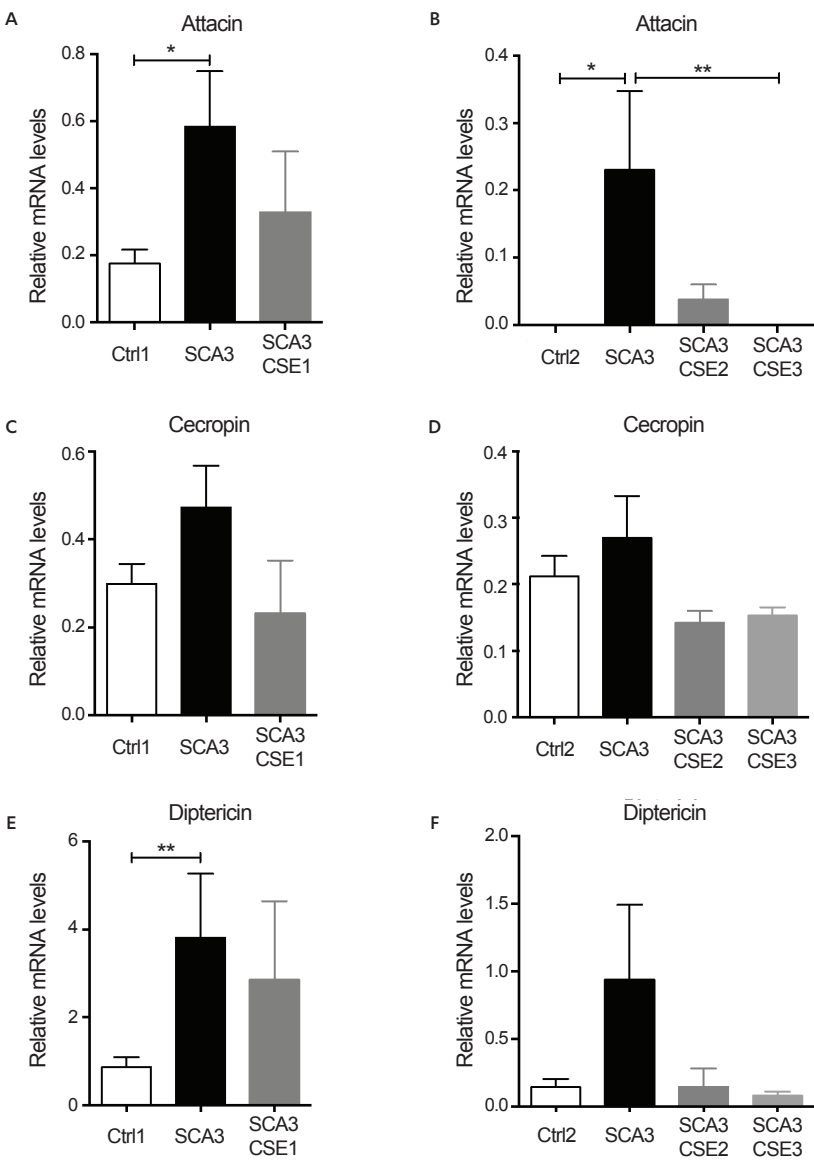


**Supplementary Figure 2. Overexpression of CSE increases protein persulfidation in the wild type background and partly rescues decreased protein persulfidation of SCA3 flies**

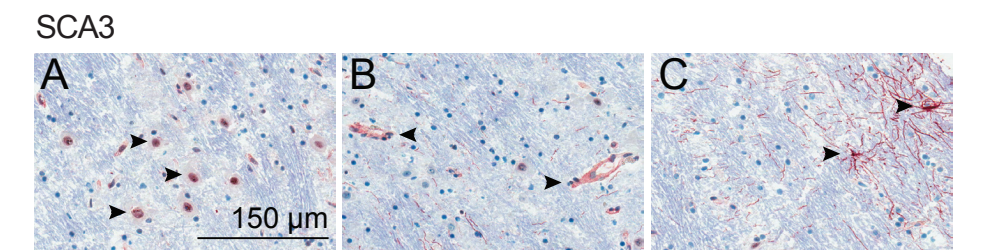
**A and B.** Protein persulfidation levels in the SCA3 fly heads are decreased compared to control flies. Protein persulfidation was determined using the biotin-labeling assay. Tubulin staining was shown to demonstrate equal protein loading of the samples. Extracts of control flies and SCA3 flies were loaded. Two blots represent two biological replications. **C.** CSE overexpression in wild type flies elevates levels of protein persulfidation, which is higher in CSE-3B than in CSE-2B overexpressing line corresponding with the mRNA levels of CSE overexpression. Protein persulfidation in **(A-C)** was determined using the biotin-labeling assay. Tubulin staining was shown to demonstrate equal protein loading of the samples.



**Supplementary Figure 3. Overexpression of CSE in a SCA3 background SCA is not associated with a decrease in insoluble/soluble fraction ratio of SCA3tr-78 protein**  
**A-D.** Set of Western blots used for analysis of fly heads and used for quantification of Figure 6B of the insoluble/soluble fraction ratio. The samples were analyzed for the amount of SCA3tr78 protein and its aggregation using an anti-HA antibody. Alpha-tubulin was used as a loading control. In SCA3 flies, both soluble monomer (in the resolving gel) and aggregated protein (in the stacking gel) fractions of SCA3tr78 protein were detected. Neither the expression levels, nor the insoluble/soluble ratio of the mutant protein were majorly modified by overexpression of CSE.



**Supplementary Figure 4. Inflammation genes that are variously affected by CSE overexpression in a SCA3 background**  
Expression of inflammation genes from the Relish pathways upon the overexpression of SCA3 alone or in combination with CSE.  
**A and B.** Attacin is induced by SCA3 but not differently expressed in the CSE-1A line. **B.** In the CSE-3B line, Attacin mRNA levels are reduced compared to w1118-B SCA3. **C and D.** Cecropin is not significantly influenced by CSE overexpression. **E.** Diptericin is significantly induced in the w1118-A background but not in the **(F)** w1118-B background. **E and F.** In all CSE overexpression lines, mRNA levels of Diptericin are not different from the isogenic control lines.



**Supplementary Figure 5. Localization pattern of CSE is not affected in pontine tissue of SCA3 patients**  
Immunohistochemistry using an anti-human CSE antibody revealed that in pontine tissue of an additional SCA3 patients (sample 4 Suppl. Table 1) (representative images are shown in A-C), CSE is localized in (A) neurons of the pontine nuclei, (B) the vasculature and (C) astrocytes. Black arrows indicate the mentioned structures.

**Supplementary Table 1. Characteristics of the SCA3 and control patients**

Patient	Age	Gender	Cause of death	CAG repeats
Control 1	79	female	metastasized lung carcinoma	-
Control 2	53	male	metastasized signet-ring carcinoma	-
Control 3	16	female	ALL + sepsis	-
Control 4	17	male	lung transplantation + alveolar hemorrhage	-
Control 5	83	male	contusio cerebri	-
Control 6	27	male	car accident	-
Control 7	58	male	bronchopneumonia + lung emboli	-
SCA3 1	70	female	-	68
SCA3 2	63	male	-	70
SCA3 3	38	male	-	81
SCA3 4	71	male	-	70
SCA3 5	88	female	-	65
SCA3 6	34	female	-	77